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Carboxylesterase-mediated insecticide resistance: Quantitative increase induces broader metabolic resistance than qualitative change

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ABSTRACT

Carboxylesterases are mainly involved in the mediation of metabolic resistance of many insects to organophosphate (OP) insecticides. Carboxylesterases underwent two divergent evolutionary events: (1) quantitative mechanism characterized by the overproduction of carboxylesterase protein; and (2) qualitative mechanism caused by changes in enzymatic properties because of mutation from glycine/alanine to aspartate at the 151 site (G/A151D) or from tryptophan to leucine at the 271 site (W271L), following the numbering of *Drosophila melanogaster* AChE. Qualitative mechanism has been observed in few species. However, whether this carboxylesterase mutation mechanism is prevalent in insects remains unclear. In this study, wild-type, G/A151D and W271L mutant carboxylesterases from *Culex pipiens* and *Aphis gossypii* were subjected to germline transformation and then transferred to *D. melanogaster*. These germlines were ubiquitously expressed as induced by *tub*-Gal4. In carboxylesterase activity assay, the introduced mutant carboxylesterase did not enhance the overall carboxylesterase activity of flies. This result indicated that G/A151D or W271L mutation disrupted the original activities of the enzyme. Less than 1.5-fold OP resistance was only observed in flies expressing *A. gossypii* mutant carboxylesterases compared with those expressing *A. gossypii* wild-type carboxylesterase. However, transgenic flies universally showed low resistance to OP insecticides compared with non-transgenic flies. The flies expressing *A. gossypii* W271L mutant esterase exhibited 1.5-fold resistance to deltamethrin, a pyrethroid insecticide compared with non-transgenic flies. The present transgenic *Drosophila* system potentially showed that a quantitative increase in carboxylesterases induced broader resistance of insects to insecticides than a qualitative change.

1. Introduction

The evolution of insecticide resistance in insect pests as a consequence of strong selection imposed by widespread insecticide application impedes pest control efforts [1]. Chemical insecticides are the preferred agents used to control numerous agricultural and vector insect pests. Organophosphate (OP) insecticides are among the most commonly used insecticides since organochlorine insecticides were banned. However, many insect pests have developed resistance to OPs (http://www.pesticideresistance.org).

Resistance to OP insecticides can be mediated either by exhibiting insensitivity as a result of target enzyme (acetylcholinesterase) mutations or by enhancing the ability to metabolize OPs. Studies on metabolic-based insecticide resistance have provided valuable insights into the mechanism by which insecticides are inactivated before molecular targets in insects are reached. This metabolic resistance to OP insecticides is mediated by carboxylesterases (EC 3.1.1.1), particularly nonspecific carboxylesterases, which exhibit high activity (as wild-type enzymes) with substrates, such as α- or β-naphthyl-acetate. An individual insect species may contain large numbers of carboxylesterase genes; for example, *Bombyx mori* contains 76 carboxylesterase genes [2]. Despite this large number, only several carboxylesterase genes are usually responsible for OP resistance. For instance, two closely located carboxylesterase genes on chromosome II of *Culex pipiens*, namely,

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Est-3 (coding esterase A) and *Est-2* (coding esterase B), provide a substantial level of OP resistance in field populations worldwide [3,4]. E4 and FE4 esterases in *Myzus persicae* also exhibit resistance to OP [5].

Two metabolic resistance mechanisms are involved in nonspecific carboxylesterases. Quantitative mechanism is characterized by the overproduction of carboxylesterase proteins by gene amplification or transcriptional upregulation. In this mechanism, high-level esterase proteins function as "sponges" to sequester insecticide molecules, not to hydrolyze these substances [6]. Lower dipterans, such as *C. pipiens, Culex tarsalis*, and *Culex tritaeniorhynchus*, and sap-sucking insects, such as *M. persicae* and *Schizaphis graminum*, utilize this strategy to survive OP treatments [3,7–9]. Qualitative mechanism occurs as a result of changes in the enzymatic properties of esterases, specifically high activities with OPs and low activities with common substrates, such as naphthyl acetate. The same mutant form G137D (denoted as G151D in this paper, following the sequence of *Drosophila melanogaster* AChE), which is found in field-resistant populations of several higher dipterans [e.g., *Lucilia cuprina* (LcαE7), *Lucilia sericata* (LsαE7), *Cochliomyia hominivorax*, and *Musca domestica* (MdαE7)], causes the characteristic shift in substrate specificity from naphthyl acetate to OPs [10–13]. W251L (denoted as W271L in this paper, following the sequence of *D. melanogaster* AChE), another variant in malathion-resistant *L. cuprina* populations (LcαE7), exhibits a similar shift in substrate specificity. As a result, naphthyl acetate hydrolysis activity is decreased, whereas OP activity is increased [14]. W251G, a different substitution, was also found at position 251 in a malathion-resistant strain of the parasitoid wasp *Anisopteromalus calandrae* [15].

Although the qualitative mechanism has been observed in few insect pests, in vitro mutagenesis and recombinant expression of nonspecific carboxylesterases from eight insect species, including *Aphis gossypii, C. pipiens, Spodoptera litura, B. mori, Nilaparvata lugens, Tribolium castaneum, Harmonia axyridis*, and *Apis mellifera*, indicate that the G/A151D and W271L mutations commonly induce changes in the enzymatic properties of insect carboxylesterases at a biochemical level [16,17]. However, the two mutations in eight *Helicoverpa armigera* esterases do not generally enhance either OP or pyrethroid hydrolysis in vitro [18]. As such, in vivo experimental evidence should be obtained to determine whether carboxylesterase evolution via qualitative mechanism potentially causes broad insecticide resistance of insects. Studies should also be conducted to determine whether quantitative or qualitative mechanism provides insects with efficient adaptive mechanisms to insecticide selection.

In this study, wild-type, G/A151D and W271L mutant carboxylesterases of *C. pipiens* (esterase B1, M32328, named as CpEST) and *A. gossypii* (EU783916, named as AgEST) were chosen for gene transformation to *D. melanogaster* by considering the significant differences of these enzymes in insecticide hydrolysis in vitro. The A151D and W271L mutants of AgEST showed considerable hydrolysis activities toward OP insecticides, whereas only the W271L mutant CpEST had low OP hydrolysis activity [17]. The hydrolysis activities of CpEST, AgEST, and their mutants toward pyrethroid insecticides were not determined in vitro. However, 271L mutation has been reported to be capable of increasing the hydrolysis activity of other insect esterases, like *L. cuprina* E3, *D. melanogaster* EST23, and three *H. armigera* esterases, toward some pyrethroid isomer insecticides in vitro [18,19]. Therefore, in this study, insecticide resistance was investigated by comparing the carboxylesterase activities and resistance levels to four OP insecticides and one pyrethroid insecticide between (1) wild-type and mutant esterase transgenic lines and (2) between transgenic lines and non-transgenic lines. The evolutionary fitness characteristics of the two resistance mechanisms were discussed.

2. Materials and methods

2.1. Insect stocks

All of the *Drosophila* lines were maintained at 25 °C in a standard corn meal/molasses medium [20] with a 12 h/12 h light/dark cycle at 60% humidity. The following fly strains were used: w^{1118}; J36 (yw, M{eGFP,Vas-int.Dm}ZH-2A;;M{RFP.attp}ZH-86Fb); Sco/Cyo; TM3/TM6b; and *tub*-Gal4 [21]. The mosquito strain (*C. pipiens*) Shengui is an OP-resistant strain maintained at 26 ± 1 °C with a long-day photoperiod (14 h/10 h light/dark cycle) [22,23]. The cotton aphid strain (*A. gossypii*) was collected in Tai'an, Shandong, in 2010 and reared in the lab at 25 ± 1 °C with a long-day photoperiod (14 h/10 h light/dark cycle).

2.2. Transgenic fly generation

The fragments corresponding to the wild-type carboxylesterases, two mutant G/A151D and W271L forms of *CpEST* and *AgEST*, were amplified from the previously constructed recombinant pET28a plasmids [16,17] and respectively subcloned into pUAST or pUAST-attB vectors [24,25] between the restriction digestion sites *Bgl*II and *Kpn*I. The primer pairs used in the PCRs were CpEST–BglII and CpEST–KpnI and AgEST–BglII and AgEST–KpnI (Table S1). All cloning steps were confirmed by restriction digestion and DNA sequencing. The plasmids expressing wild-type CpEST or AgEST were named as p(UAS-CpEST-wildtype) or p(UAS-AgEST-wildtype); the plasmids expressing mutant CpEST or AgEST with point mutation were named as p(UAS-CpEST-G151D), p(UAS-CpEST-W271L), p(UAS-AgEST-A151D), and p(UAS-AgEST-W271L), respectively. The six recombinant plasmids were subjected to germline transformation to generate transgenic lines according to standard protocols [26].

2.3. Quantitative PCR

Quantitative PCR (qPCR) was performed to quantify the gene copy number and the RNA level of carboxylesterases between wild-type and mutant transgenic lines. Each transgenic strain was crossed with the Gal4 strain. Genomic DNA and total RNA were obtained from six independent groups of offspring by using cetyl trimethyl ammonium bromide method [27] and TRIZOL reagent (Invitrogen, Carlsbad, CA, USA), respectively. Each group comprised five 4-day-old female adult flies. The total RNA was treated using the TURBO DNA-free kit (Ambion, Austin, TX, USA) to remove genomic DNA contamination and then was reverse-transcribed into cDNA by using the SuperScript™ III first strand synthesis system (Invitrogen). The relative differences in the mRNA expression levels or inserted-gene copies among transgenic strains were quantified by qPCR (LightCycler® 480 II system; Roche, Basel, Switzerland). The *actin 42A* of *D. melanogaster* (NM_078901) was used as an internal control. The primers were designed using Primer-BLAST in NCBI (http://www.ncbi.nlm.nih.gov/tools/primer-blast) (Table S1). One-way ANOVA was performed using SPSS 13.0 to analyze relative differences. Values were presented as mean ± SEM.

2.4. Carboxylesterase activity assays

For each transgenic strain, the Gal4-driven total carboxylesterase activities of the four-day-old female adult progenies were quantified using a Beckman DU-800 spectrophotometer (Becton-Dickinson, Fullerton, CA, USA) with artificial substrate β-naphthyl-acetate in 0.2 M phosphate buffer (pH 7.0). Gross proteins extracted from the three flies were incubated with β-naphthyl-acetate for 3 min at 37 °C. Subsequently, freshly prepared diazo blue sodium dodecyl sulfate (SDS) reagent (0.3% fast blue B salt in 3.5% aqueous SDS) was added. The color that developed as a result of β-naphthol formation was detected at 555 nm and

quantified using a β-naphthol standard curve. Enzymatic activity was determined as the amount of β-naphthol (μM) produced per milligram of total protein per minute. Six biological replicates were performed, and one-way ANOVA was conducted to analyze the relative differences in enzyme activities among various fly strains.

2.5. Insecticide bioassays

Four-day-old female adult offsprings from the following hybridization groups were used in topical application bioassays of several insecticides: male transgenic flies crossed with female Gal4 lines and male transgenic flies crossed with female w^{1118}. Four OP insecticides (i.e., chlorfenvinphos, malathion, parathion, and monocrotophos) and one pyrethroid insecticide (i.e., deltamethrin) were dissolved in acetone and diluted to obtain the final concentrations. The flies were anesthetized with carbon dioxide; afterward, 0.25 μL of insecticide solution was applied on the thoracic dorsum of flies by using a Burkard hand microapplicator (Burkard Manufacturing Co., Hertfordshire, England). Control flies were treated with 0.25 μL of acetone. Five doses of each insecticide with six replicates for each dose (20 flies per replicate) were administered. Mortality was determined at 24 h after treatment, and data were analyzed using the log-probit program [28,29] to obtain LD_{50} values, slope of each mortality line, Chi-square values, and resistance ratios with 95% confidence intervals.

2.6. Probe synthesis

A 201 bp fragment of *CpEST* and a 262 bp fragment of *AgEST* were amplified from the previously constructed recombinant pET28a plasmids [16,17] by using the following primers: CpEST-probe-For and CpEST-probe-Rev for *CpEST*; and AgEST-probe-For and AgEST-probe-Rev for *AgEST* (Table S1). These fragments were subcloned into the pGEM-T vector (Promega, Madison, WI, USA). The plasmids were confirmed by sequencing and linearized with *Sac*I or *Apa*I to perform transcription with T7 or SP6 RNA polymerase and to generate digoxigenin (DIG)-labeled antisense and sense probes by using a DIG RNA labeling kit (Roche). These probes were evaluated by dot-blot hybridization according to the manufacturer's instructions.

2.7. In situ hybridization

Adult insects of mosquitoes and cotton aphids were immobilized in an ice bath to remove their wings and legs. Two holes were created in the thorax and the abdomen. The insects were then fixed overnight in 4% paraformaldehyde containing 0.25% triton X-100. The paraffin-embedded specimens were sliced with a thickness of 5 μm, deparaffinized, and further digested with 20 μg/mL proteinase K. Afterward, the sections were hybridized using DIG-labeled antisense and sense probes at 55 °C for 19 h in a hybridization incubator. For detection, the sections were incubated with anti-DIG-AP conjugate (Roche; 1:1000) at 37 °C for 2 h and visualized using the nitro blue tetrazolium/5-bromo-4-chloro-3-indolylphosphate colorimetric substrates (Promega) according to the manufacturer's instructions. Photographs were obtained using an inverted microscope (ECLIPSE TE2000-S; Nikon).

3. Results

3.1. Ubiquitous carboxylesterase gene expressions in C. pipiens and A. gossypii

The distributions of the carboxylesterase CpEST and AgEST were examined by *in situ* hybridization in *C. pipiens* and *A. gossypii*, respectively, to evaluate whether or not the ubiquitous *tub*-Gal4 line should be used in later transgenic experiments. CpEST was expressed in the brain, eyes, ovary, nerve ganglion, digestive gut, and salivary gland of mosquitoes (Fig. 1). AgEST was expressed in the brain, nerve ganglion, digestive gut, ovary, muscle, and cuticle of aphids (Fig. 2). These two carboxylesterases in the two insect species showed a pervasive, not organ-specific expression pattern. Thus, the *tub*-Gal4 line was chosen to drive the expression of the two carboxylesterases ubiquitously in *Drosophila*.

3.2. Comparative gene copy number and transcript levels of the transformed carboxylesterase genes in transgenic Drosophila strains

The wild-type, G/A151D and W271L mutant CpEST and AgEST were subjected to germline transformation to the third chromosome of *D. melanogaster*. The DNA and RNA levels of the transferred carboxylesterase genes in these transgenic lines were determined by real-time qPCR. At the DNA level, the inserted-gene copy numbers of the wild type and the two mutants were similar in CpEST (Fig. 3A) and AgEST (Fig. 3B) transgenic lines. At carboxylesterase transcript levels, no significant difference was observed in the carboxylesterase expression induced by *tub*-Gal4 of wild-type and mutant transgenic lines (Fig. 3C and D, $P > 0.05$ in one-way ANOVA). This uniformity in the gene copy and transcript levels for the wild-type and the corresponding mutant transgenic lines made the comparison of carboxylesterase activity and insecticide resistance level among these transgenic strains rational. No transcripts (leaky expression) of the transferred carboxylesterase genes were detected from UAS lines before crossing to *tub*-Gal4.

3.3. Decreased carboxylesterase activity in mutant esterase transgenic flies

The overall *tub*-Gal4-driven carboxylesterase activities in the transgenic flies were determined to evaluate the activities of the two mutant carboxylesterases. The carboxylesterase activities of the wild-type CpEST and wild-type AgEST transgenic flies to β-naphthyl-acetate were approximately twofold higher than those of 151D or 271L mutant transgenic flies and non-transgenic flies (Fig. 4A and B). No significant difference was observed between 151D or 271L mutant transgenic flies and non-transgenic flies. Thus, overexpression of 151D or 271L mutant carboxylesterase cannot increase the overall carboxylesterase activity in flies, indicating that G/A151D or W271L mutation disrupts the β-naphthyl-acetate hydrolysis activity of enzymes. The results of CpEST-151D, CpEST-271L, and AgEST-271L are consistent with those obtained from in vitro expressed enzymes, whereas the AgEST-151D mutant is not behaving in the fly as the in vitro expressed enzyme, which biochemically shows similar β-naphthyl-acetate hydrolysis activity as the wild-type AgEST [17].

3.4. Mutant A. gossypii esterases exhibit low OP resistance

To determine whether mutant carboxylesterases were involved in insecticide resistance, we evaluated the resistance levels of transgenic flies to four organophosphorus and one pyrethroid insecticides, in which exogenous wild-type or mutant carboxylesterases were expressed by the *tub*-Gal4 promoter. CpEST-151D or CpEST-271L transgenic flies did not show higher resistance levels to the tested insecticides. Instead, these flies were more susceptible to parathion, malathion, or deltamethrin than the wild-type CpEST transgenic flies were ($P \leq 0.05$) (Table 1). The AgEST-151D mutant flies significantly exhibited 1.4-fold higher resistance to chlorfenvinphos ($P < 0.05$) and 1.2-fold marginally significant resistance to monocrotophos ($P = 0.05$) (Table 2) compared with the wild-type AgEST flies. The AgEST-271L mutant flies were also 1.2-fold marginally resistant to chlorfenvinphos ($P = 0.05$) (Table 2). No significant higher resistance to the three other insecticides were observed in AgEST-151D or AgEST-271L mutant flies. However, AgEST-151D and AgEST-271L flies were more susceptible to parathion, and AgEST-151D was more susceptible to malathion than the wild-type AgEST transgenic flies was ($P \leq 0.05$) (Table 2).

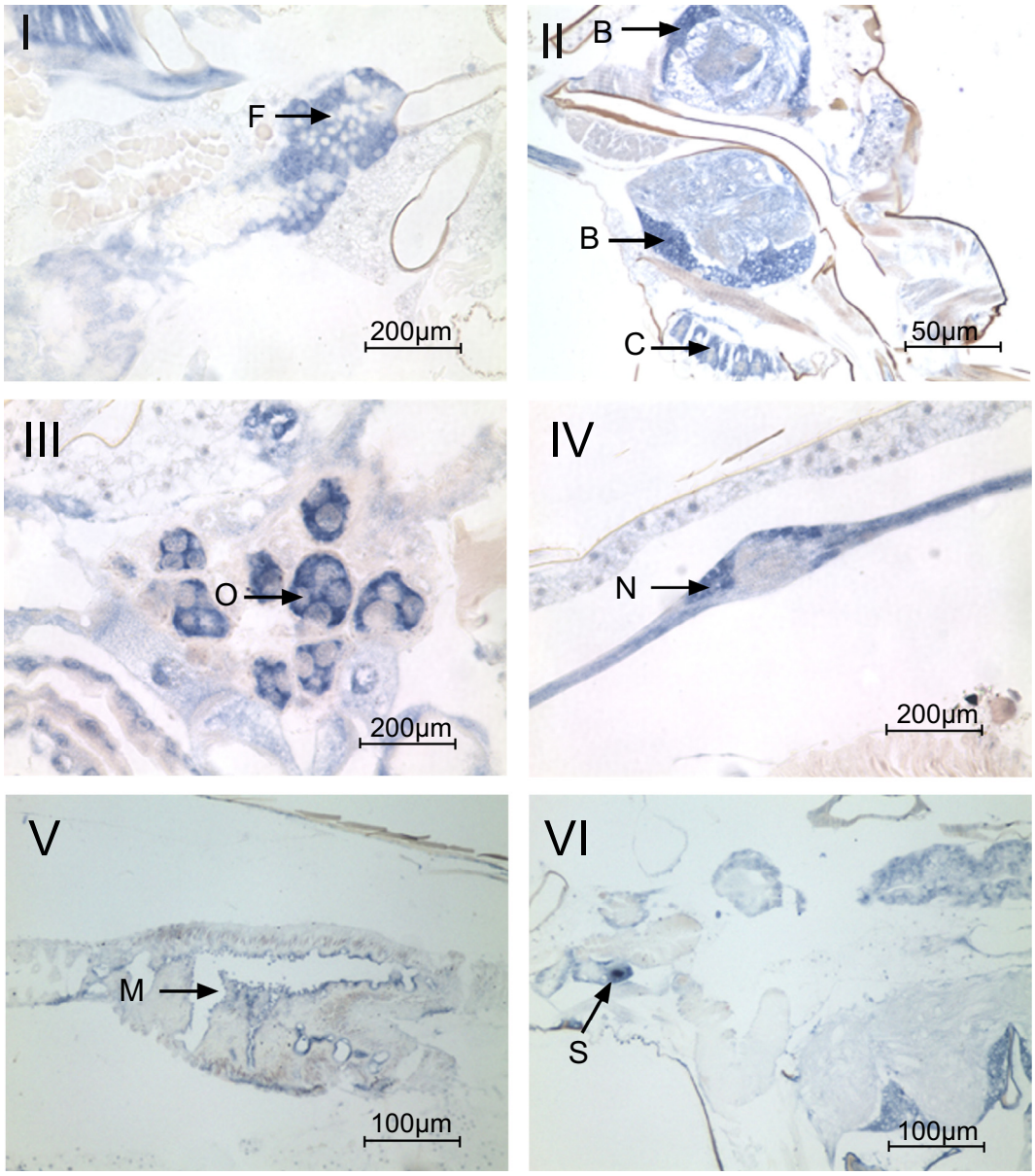

Fig. 1. Expression pattern of CpEST in *Culex pipiens* adults detected by in situ hybridization. The positive signal (antisense probe) was in blue. The negative control (sense probe) did not show positive staining. F, Foregut. B, Brain. C, Compound eye. O, Ovary. N, Nerve cord. M, Midgut. S, Salivary gland. (For interpretation of the references to color in this figure legend, the reader is referred to the web version of this article.)

3.5. Quantitative increase in carboxylesterases enhances insecticide resistance

We evaluated the effects of quantitative increase in carboxylesterases on insecticide resistance level by comparing the responses of transgenic lines before and after crossing to *tub*-Gal4 (meaning overexpression of the transferred genes). The overexpression of wild-type *C. pipiens* esterase induced 1.4-fold to 2.0-fold higher resistance ($P \leq 0.05$) to chlorfenvinphos, parathion, or monocrotophos, but no resistance to malathion or deltamethrin was observed (Table 1). CpEST-151D-overexpressed flies displayed comparative resistance to chlorfenvinphos and malathion, whereas CpEST-271L-overexpressed flies exhibited resistance to chlorfenvinphos and parathion ($P < 0.05$) (Table 1). The overexpressions of wild-type or AgEST-151D mutant *A. gossypii* esterases caused 1.3-fold to 2.5-fold resistance to the four OP insecticides. By contrast, the overexpression of AgEST-271L mutant esterase induced resistance to three OP insecticides and 1.5-fold resistance to deltamethrin, a pyrethroid insecticide ($P \leq 0.05$) (Table 2). These results indicated that insecticide resistance could be attributed to quantitative increase in carboxylesterase levels.

4. Discussion

Metabolic resistance to OP insecticides mediated by nonspecific carboxylesterases is an important mechanism that has evolved in insects because of insecticide selection. Two point mutations, namely, G151D and W271L, cause OP resistance by altering the enzymatic properties of nonspecific carboxylesterases in few insect species. In this study, the exogenous wild-type, G/A151D or W271L mutant carboxylesterase genes of two insects, particularly *C. pipiens* and *A. gossypii*, were transformed to the *Drosophila* genome. G/A151D or W271L mutation disrupted the original activities of the two carboxylesterases to β-naphthyl-acetate. Only the mutant AgEST

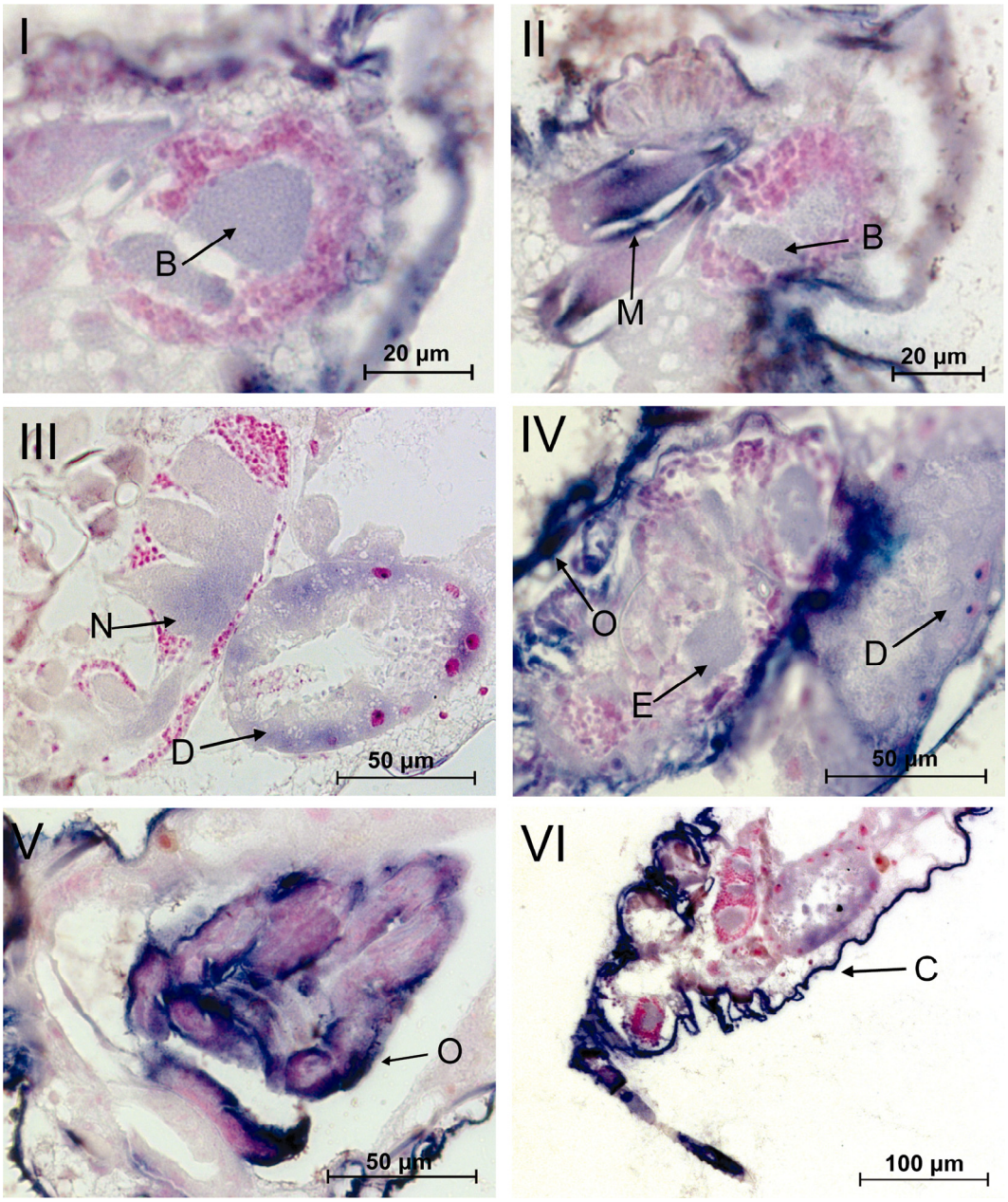

Fig. 2. Expression pattern of AgEST in *Aphis gossypii* adults detected by in situ hybridization. The positive signal (antisense probe) was in blue. The negative control (sense probe) did not show positive staining. Nuclei were stained in red with Nuclear Fast Red. B, Brain. M, Muscle. N, Nerve node. D, Digestive gut. O, Ovary. E, Embryo. C, Cuticle. (For interpretation of the references to color in this figure legend, the reader is referred to the web version of this article.)

transgenic flies exhibited marginally higher resistance to monocrotophos and/or chlorfenvinphos compared with the wild-type AgEST transgenic flies. However, the overexpression of any of the two insect carboxylesterases in flies caused significant higher insecticide resistance. These results demonstrated that quantitative mechanism provided insects with more efficient adaptive responses to insecticide selection than qualitative mechanism.

Quantitative mechanism is more commonly used by insects than qualitative mechanism when they are confronted with selection pressure from exposure to insecticides. This response commonly occurs probably because of the lower fitness cost of quantitative resistance mechanism. For example, the overproduction of esterase by the *Ester* locus (*Ester*^{4} and *Ester*^{1}) in *C. pipiens* increases the probability of predation and developmental time [30]; this condition also decreases mating competition among males [31]. The alteration of fitness-related traits in qualitative resistance mechanism is likely harsher. Diazinon- and malathion-resistant *L. cuprina* characterized by G151D and W271L mutations in E3 exhibits an increase in fluctuating asymmetry, particularly morphological left–right differences in specific bristle characters [32,33]. Fluctuating asymmetry corresponds to developmental instability and possibly causes selective disadvantages. This decrease in genetic fitness is destructive such that background modifier genes are subsequently selected to prevent the negative effects of resistance genes [32,34,35]. The in vivo functions of *D. melanogaster α-Esterase-7*, which encodes the homolog of *L. cuprina* E3, have been detected in a deletion mutant fly strain by gene targeting. *D. melanogaster α-Esterase-7* not only provides wild-type flies with moderate insecticide tolerance but also influences the lipid metabolism and lifespan of flies [36].

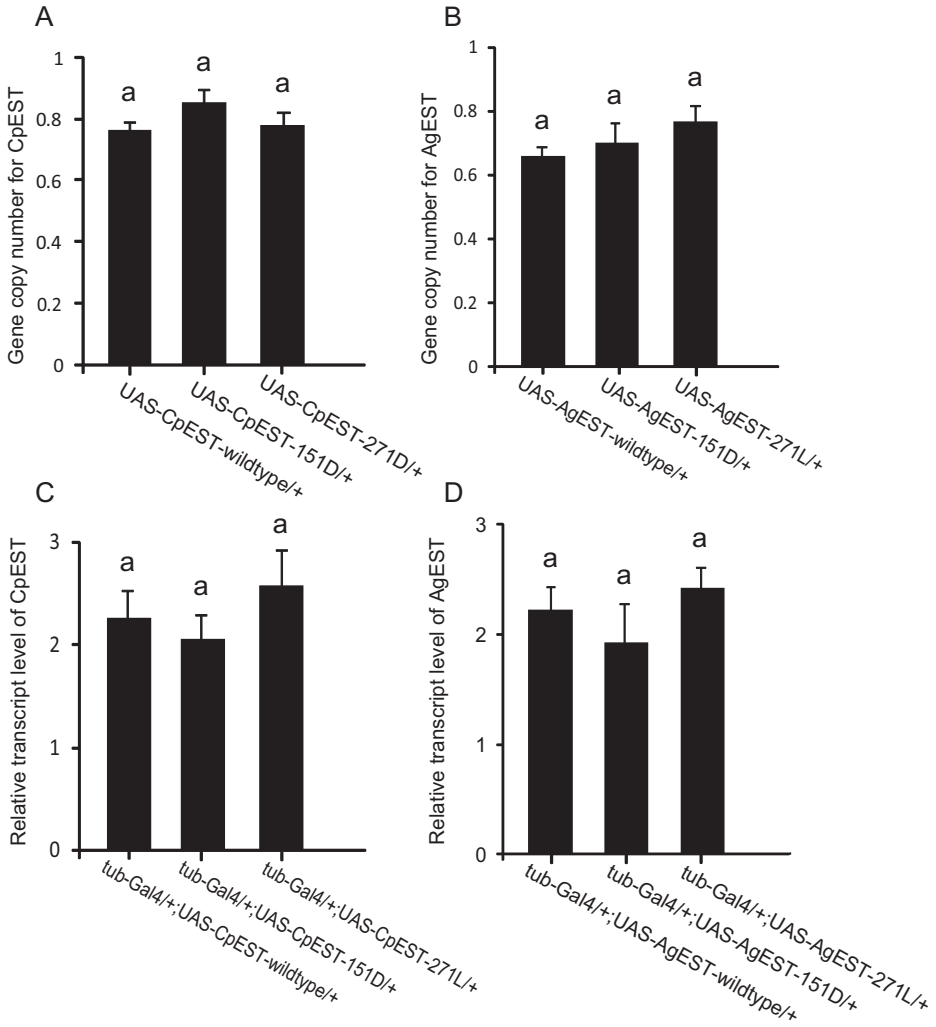

Fig. 3. Gene copy numbers of CpEST and AgEST inserted in *Drosophila* genome and the relative transcript levels in various transgenic lines driven by *tub*-Gal4 detected by qPCR. (A) and (B) indicate the inserted gene copy numbers of CpEST and AgEST in the *Drosophila* genome, respectively. The gene copy number smaller than 1 is presumably attributed to less than 100% of chromosome transformation. (C) and (D) indicate the relative transcript levels of CpEST and AgEST normalized by the transcript level of *Drosophila melanogaster actin 42A* (NM_078901), respectively. The same letter indicates no significant difference ($P > 0.05$) among the three genotypes of flies. Error bars indicate SEM.

D. melanogaster is an in vivo system that has been used to validate candidate resistance genes and is applicable to many detoxification gene families from different species, such as various P450 genes from *Drosophila* [37,38], P450 *Cyp6d1* from *M. domestica* [39], P450 *Cyp6bq9* from *T. castaneum* [40], carboxylesterase gene *αE7* from *L. cuprina*, glutathione S-transferase gene *GstE2* from *Anopheles gambiae*, and P450 *Cyp6cm1* from *Bemisia tabaci* [41]. Among the advantages of this system is a uniform genetic background. Therefore, any differences in survival from insecticide exposure are possibly attributed directly to transgene. The insecticide-resistant phenotypes observed in the transgenic *Drosophila* system resemble those observed in resistant pest species populations; however, the resistance levels in the *Drosophila* system are lower than those documented in pest species [41]. The overexpression of *C. pipiens* or *A. gossypii* esterases in *Drosophila*, no matter wild-type or mutant enzymes, produced at most 2.5-fold resistance to OP insecticides in this study, much lower than the resistance levels generally associated with the gene amplifications seen in the aphids and mosquitoes [4,7,9]. Therefore, the insecticide resistance levels observed in this study could be underestimated if the mutations were observed in field populations of *C. pipiens* or *A. gossypii*. Another possible reason for this underestimation is that the resistance levels presented in this study were manifested in heterozygous flies or those with a mutant allele and a wild-type allele as a result of the cross between transgenic flies and Gal4 flies.

The insecticide-resistant phenotypes of the mutated carboxylesterases from *C. pipiens* and *A. gossypii* presented in this study differed from those of *L. cuprina* E3. The G151D mutation of E3 generated 10-fold to 16-fold increase in resistance to diethyl OP diazinon; by contrast, no evident resistance to dimethyl OP malathion was observed. The W271L mutation of E3 resulted in 3-fold to 5-fold resistance to diazinon and 600-fold resistance to malathion in *L. cuprina* [14]. No evident resistance to the tested insecticides was induced by G151D or W271L mutation of *C. pipiens* carboxylesterase. The A151D mutation of *A. gossypii* carboxylesterase induced comparable resistance to diethyl OP chlorfenvinphos and dimethyl OP monocrotophos. The W271L mutation caused only marginal resistance to chlorfenvinphos. Such different insecticide-resistant phenotypes could be attributed to species specificity or large phylogenetic differences between the two esterases (*C. pipiens* esterase B1 and *A. gossypii* esterase EU783916) and *L. cuprina* E3 [17]. However, mutations in carboxylesterases in these systems resulted in insecticide resistance.

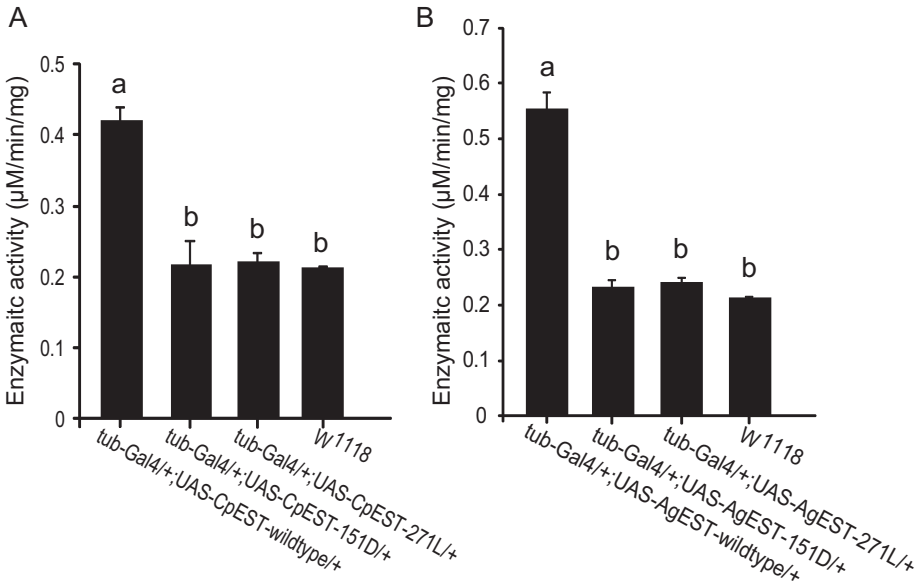

Fig. 4. Carboxylesterase activity quantified in a Beckman DU-800 spectrophotometer for transgenic flies after crossed with the *tub*-Gal4 strain. (A) CpEST transgenic strains. (B) AgEST transgenic strains. Enzymatic activity was determined as the amount of β-naphthol (μM) produced per milligram of total proteins per minute. Letters indicate the comparison among the four genotypes of flies. Error bars indicate SEM.

The resistance phenotype to malathion and parathion from transgenic flies is not consistent with that of in vitro expressed enzymes. CpEST-151D transgenic flies were more susceptible to parathion, and CpEST-271L transgenic flies were more susceptible to malathion and parathion than the wild-type CpEST transgenic flies, whereas the mutant CpEST enzymes showed either no or increased hydrolysis activities to malathion and methyl parathion in vitro [16]. Both AgEST-151D and AgEST-271L transgenic flies were more

Table 1
Insecticide resistance levels of *Culex pipiens* esterase transgenic flies.

Strain genotype and insecticide	LD_{50} (95% CI) (μg/g)	X^2	Slope (SE)	RR1 (95% CI)	RR2 (95% CI)
Chlorfenvinphos					
tub-Gal4/+;UAS-CpEST-wildtype/+	2.3(2.1–2.5)	0.1	6.4(0.8)	1	1.6*(1.2–2.5)
tub-Gal4/+;UAS-CpEST-151D/+	2.6(2.4–2.9)	2.5	5.4(0.6)	1.1 (0.8–1.6)	1.7*(1.3–2.4)
tub-Gal4/+;UAS-CpEST-271L/+	2.5(2.3–2.7)	3.3	4.9(0.5)	1.1 (0.8–1.5)	1.8*(1.2–2.3)
UAS-CpEST-wildtype/+	1.4(1.0–1.8)	3.8	1.7(0.3)		1
UAS-CpEST-151D/+	1.5(1.2–1.8)	1.1	2.1(0.3)		1
UAS-CpEST-271L/+	1.4(1.2–2.0)	5.0	1.9(0.3)		1
Malathion					
tub-Gal4/+;UAS-CpEST-wildtype/+	6.7(6.2–7.2)	4.7	6.2(0.7)	1	1.1 (0.9–1.2)
tub-Gal4/+;UAS-CpEST-151D/+	6.8(6.4–7.1)	4.5	6.1(0.5)	1.0 (0.8–1.3)	1.4*(1.1–1.7)
tub-Gal4/+;UAS-CpEST-271L/+	5.3(4.3–6.0)	0.7	4.2(0.7)	0.8*(0.6–1.0)	1.2 (0.9–1.4)
UAS-CpEST-wildtype/+	6.3(6.0–6.7)	4.2	8.9(0.9)		1
UAS-CpEST-151D/+	4.8(4.2–5.7)	0.7	4.0(0.6)		1
UAS-CpEST-271L/+	4.5(4.2–4.7)	1.3	9.1(1.3)		1
Parathion					
tub-Gal4/+;UAS-CpEST-wildtype/+	3.0(2.8–3.1)	3.7	9.8(1.2)	1	2.0*(1.9–2.1)
tub-Gal4/+;UAS-CpEST-151D/+	1.6(1.5–1.7)	0.2	6.2(0.6)	0.5*(0.4–0.7)	0.9 (0.8–1.1)
tub-Gal4/+;UAS-CpEST-271L/+	1.8(1.6–1.9)	4.6	12.9(2.4)	0.6*(0.4–0.9)	1.4*(1.1–1.5)
UAS-CpEST-wildtype/+	1.5(1.4–1.5)	5.6	11.8(1.4)		1
UAS-CpEST-151D/+	1.8(1.6–2.0)	2.8	5.3(0.7)		1
UAS-CpEST-271L/+	1.3(1.3–1.4)	2.4	17.6(2.5)		1
Monocrotophos					
tub-Gal4/+;UAS-CpEST-wildtype/+	3.7(3.4–4.0)	0.5	7.0(1.2)	1	1.4*(1.0–1.8)
tub-Gal4/+;UAS-CpEST-151D/+	3.1(2.7–3.4)	1.1	5.4(0.9)	0.8 (0.6–1.2)	0.9 (0.7–1.2)
tub-Gal4/+;UAS-CpEST-271L/+	3.5(3.2–3.8)	3.2	7.4(1.2)	0.9 (0.7–1.4)	1.1 (0.9–1.4)
UAS-CpEST-wildtype/+	2.7(2.2–3.4)	0.9	4.2(0.8)		1
UAS-CpEST-151D/+	3.4(2.9–3.8)	1.1	4.0(0.7)		1
UAS-CpEST-271L/+	3.1(2.8–3.4)	1.0	5.6(0.9)		1
Deltamethrin					
tub-Gal4/+;UAS-CpEST-wildtype/+	4.1(3.7–4.5)	0.2	5.2(0.5)	1	0.8 (0.6–1.0)
tub-Gal4/+;UAS-CpEST-151D/+	3.1(2.7–3.4)	7.1	2.6(0.2)	0.8*(0.6–1.0)	0.6 (0.4–0.8)
tub-Gal4/+;UAS-CpEST-271L/+	4.1(3.8–4.4)	2.1	3.6(0.2)	1.0 (0.8–1.3)	1.0 (0.8–1.2)
UAS-CpEST-wildtype/+	5.1(4.6–5.7)	1.6	4.0(0.5)		1
UAS-CpEST-151D/+	5.4(4.3–6.7)	2.0	1.5(0.2)		1
UAS-CpEST-271L/+	4.2(3.7–4.6)	0.1	4.4(0.5)		1

* $P \leq 0.05$; RR1, LD_{50} of *tub*-Gal4/+;UAS-CpEST-wildtype/+ divided by that of *tub*-Gal4/+;UAS-CpEST-151D/+ or *tub*-Gal4/+;UAS-CpEST-271L/+; RR2, LD_{50} of each *tub*-Gal4/+;UAS line/+ divided by LD_{50} of the corresponding parent UAS control.

Table 2
Insecticide resistance levels of *Aphis gossypii* esterase transgenic flies.

Strain genotype and insecticide	LD_{50} (95% CI) (μg/g)	X^2	Slope (SE)	RR1 (95% CI)	RR2 (95% CI)
Chlorfenvinphos					
tub-Gal4/ + ;UAS-AgEST-wildtype/ +	2.0(1.9–2.2)	2.3	8.1(0.9)	1	1.3*(1.0–1.8)
tub-Gal4/ + ;UAS-AgEST-151D/ +	2.8(2.5–3.0)	4.0	5.7(0.6)	1.4*(1.2–1.6)	1.4*(1.1–1.7)
tub-Gal4/ + ;UAS-AgEST-271L/ +	2.4(2.2–2.7)	3.3	4.5(0.5)	1.2*(1.0–1.5)	1.5*(1.0–2.1)
UAS-AgEST-wildtype/ +	1.6(1.2–2.0)	2.7	2.0(0.3)		1
UAS-AgEST-151D/ +	2.0(1.8–2.3)	1.5	3.9(0.6)		1
UAS-AgEST-271L/ +	1.6(1.3–2.1)	1.3	2.2(0.3)		1
Malathion					
tub-Gal4/ + ;UAS-AgEST-wildtype/ +	6.3(5.9–6.8)	2.8	9.7 (1.3)	1	1.5*(1.3–1.7)
tub-Gal4/ + ;UAS-AgEST-151D/ +	5.1(4.7–5.5)	1.5	8.4(1.3)	0.8 *(0.7–0.9)	1.3*(1.1–1.6)
tub-Gal4/ + ;UAS-AgEST-271L/ +	6.5(6.1–7.0)	1.8	9.0(1.2)	1.0 (0.9–1.2)	1.7*(1.5–2.1)
UAS-AgEST-wildtype/ +	4.3(4.0–4.6)	2.4	6.3(0.7)		1
UAS-AgEST-151D/ +	3.8(3.5–4.2)	0.1	5.2(0.6)		1
UAS-AgEST-271L/+	3.8(3.4–4.1)	0.7	5.6(0.7)		1
Parathion					
tub-Gal4/ + ;UAS-AgEST-wildtype/ +	2.5(2.3–2.7)	0.4	6.6(0.9)	1	2.5*(1.8–3.4)
tub-Gal4/ + ;UAS-AgEST-151D/ +	1.9(1.7–2.1)	2.8	5.7(0.7)	0.8* (0.7–0.9)	1.4*(1.1–1.9)
tub-Gal4/ + ;UAS-AgEST-271L/ +	2.0(1.9–2.2)	1.3	6.9(0.8)	0.8* (0.7–1.0)	2.0*(1.5–3.1)
UAS-AgEST-wildtype/ +	1.0(0.8–1.3)	1.9	1.9(0.3)		1
UAS-AgEST-151D/ +	1.4(1.1–1.6)	0.6	4.3(0.7)		1
UAS-AgEST-271L/ +	1.0(0.7–1.3)	1.5	1.7(3.0)		1
Monocrotophos					
tub-Gal4/ + ;UAS-AgEST-wildtype/ +	2.7(2.3–3.0)	0.0	5.6(0.6)	1	1.6*(1.3–2.0)
tub-Gal4/ + ;UAS-AgEST-151D/ +	3.2(2.9–3.4)	0.6	9.7(1.3)	1.2*(1.0–1.5)	1.7*(1.4–2.0)
tub-Gal4/ + ;UAS-AgEST-271L/ +	2.5(2.3–2.7)	2.6	5.6(0.6)	0.9 (0.8–1.2)	1.0 (0.9–1.2)
UAS-AgEST-wildtype/ +	1.7(1.5–1.8)	0.2	5.4(0.7)		1
UAS-AgEST-151D/ +	1.9(1.7–2.1)	2.4	5.1(0.6)		1
UAS-AgEST-271L/ +	2.4(2.2–2.6)	2.8	5.1(0.5)		1
Deltamethrin					
tub-Gal4/ + ;UAS-AgEST-wildtype/ +	3.0(2.6–3.3)	1.2	4.0(0.4)	1	1.1 (0.8–1.4)
tub-Gal4/ + ;UAS-AgEST-151D/ +	3.3(2.9–3.9)	0.0	3.5(0.5)	1.1 (0.9–1.5)	1.3 (0.9–2.1)
tub-Gal4/ + ;UAS-AgEST-271L/ +	3.1(2.7–3.5)	0.1	4.1(0.5)	1.0 (0.8–1.3)	1.5*(1.1–1.9)
UAS-AgEST-wildtype/ +	2.8(2.3–3.3)	0.1	2.9(0.6)		1
UAS-AgEST-151D/ +	2.6(1.9–3.4)	2.0	1.9(0.4)		1
UAS-AgEST-271L/ +	2.1(1.8–2.5)	0.9	3.0(0.4)		1

* $P \leq 0.05$; RR1, LD_{50} of *tub*-Gal4/ + ;UAS-AgEST-wildtype/ + divided by that of *tub*-Gal4/ + ;UAS-AgEST-151D/ + or *tub*-Gal4/ + ;UAS-AgEST-271L/ + ; RR2, LD_{50} of each *tub*-Gal4/ + ;UAS line/ + divided by LD_{50} of the corresponding parent UAS control.

susceptible to parathion while the two mutations conferred hydrolysis activity to paraoxon (the effective form of parathion) for AgEST in vitro [17]. This inconsistency between in vivo and in vitro experimental results could arise from multiple reasons, one of which obviously is the controllability of hydrolysis reaction in vitro and the complex integrative effects of an exogenous gene knock-in.

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Appendix: Supplementary material

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